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(71) Applicant *(for all designated States except US)*: THE UNIVERSITY OF BRITISH COLUMBIA [CA/CA]; University-Industry Liaison Office, IRC Room 331, 2194 Health Sciences Mall, Vancouver, British Columbia V6T 1Z3 (CA).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: DELCARDAYRE, Stephen, B. [US/CA]; #120-2730 Acadia Road, Vancouver, British Columbia V6T 1R9 (CA). DAVIES, Julian, E. [GB/CA]; 4428 West Sixth Avenue, Vancouver, British Columbia V6R 1V3 (CA).

(74) Agent: ROBINS, Roberta, L.; Robins & Associates, Suite 200, 90 Middlefied Road, Menlo Park, CA 94025 (US).

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ATGCCCaAAATAGTCGTAGTCGGAGCAGTCGCTGGTGGTGCAACATGTGCCAGCCAAATTCGACGTTTAGATAAAGAAAGTGACATT
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CTAACTGTAgATGAGTTAACTGAGTTTGAAGTGGCTTATGCACCACCATATAGCCACCCTAAAGATTTAATCAATATGATTGGTTAC
AAAGCTAAAtAA

(57) Abstract

An isolated and purified *Staphylococcus aureus* Coenzyme A disulfide reductase (CoADR) is provided. Oligonucleotides encoding the CoADR, vectors and host cells containing such oligonucleotides are also provided. In addition, antibodies reactive with the CoADR are provided, as are methods of isolating the CoADR, producing recombinant CoADR, using CoADR for screening compounds for CoADR-modulating activity, and detecting *S. aureus* in a test sample.

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STAPHYLOCOCCUS AUREUS COENZYME A DISULFIDE REDUCTASE, AND INHIBITORS THEREOF USEFUL AS ANTIMICROBIAL AGENTS

Technical Field

This invention relates generally to microbial metabolism and antimicrobial therapeutic agents. In particular, the invention relates to a novel enzyme that plays an important role in the metabolism of a number of microorganisms, including *Staphylococcus aureus*, to compounds that inhibit this enzyme, and to the use of these compounds as antimicrobial agents, particularly for the therapy of infections caused by Gram-positive organisms, especially *Staphylococcus* spp. and *Enterococcus* spp. infections.

Background

Glutathione (GSH; g-glutamyl-cysteinyl-glycine) is the predominant thiol produced by aerobic eukaryotes and Gram-positive bacteria. It is believed to protect aerobic organisms from oxygen toxicity and to participate in a multitude of functions. GSH acts as a slowly autooxidizing reserve of cysteine and as a cofactor in the detoxification of peroxides, epoxides, and other products resulting from reaction with oxygen. It is a cofactor in the reduction of disulfides and ribonucleotides and in the isomerization of protein disulfides. Thiols are the most reactive nucleophiles in the cell at physiological pH, and when exposed to atmospheric oxygen are oxidized to disulfides (RSH/RSSR = 10^{-16}). Glutathione reductase (GSR; E.C.1.6.4.2) catalyzes the NADPH-dependent reduction of intracellular oxidized glutathione (GSSG) and thereby maintains a reducing environment in

the cell (GSH/GSSG > 100). GSH was once thought to be ubiquitous. However, many organisms do not produce GSH but instead produce alternative thiols. Fahey et al. (1978) *J. Bacteriol.* 133:1126-1129; Fahey et al. (1991) *in* Meister (ed.) *Advances in Enzymology and Related Areas of Molecular Biology* 64:1-53 (John Wiley and Sons); Fairlamb (1989) *Parisitol.* 99S:93-112; Newton et al. (1989), *in* Vina (ed.), *Glutathione: Metabolism and Physiological Functions* pp. 69-77 (CRC Press, Boca Raton. FL); Newton et al. (1993) J. Bacteriol. *175*:2734- 2742; Sakuda et al. (1994) *Biosci. Biotechnol. Biochem.* 58:1347-1348; and Spies et al. (1994) *Eur. J. Biochem.* 224:203-213.

For example, *Staphylococcus aureus* produces Coenzyme A (CoA) as its major thiol instead of glutathione. Newton et al. (1996) *J. Bacteriol.*, in press. CoA is slightly more stable than glutathione to heavy metal-catalyzed auto-oxidation and provides a stable redox buffer similar to that provided by GSH in other organisms. *S. aureus* maintains millimolar levels of reduced CoA as its predominant thiol and, like most of the Gram-positive bacteria, essentially no GSH. Newton et al. (1996), *supra*; Newton et al. (1989), *supra*. CoA is required throughout metabolism as a cofactor in acyl transfer reactions and likely has additional functions in *S. aureus* analogous to those of GSH in other organisms.

Other organisms that utilize alternative thiols produce an enzyme analogous to GSR. The preferred substrate for such an enzyme is the disulfide of the predominant thiol in the cell. Shames et al. (1986) *Biochemistry* 25:3519-3526; Swerdlow et al. (1983) *J. Bacteriol.* 153:475-484. All such enzymes belong to a widespread family of pyridine nucleotide dependent disulfide reductases that include GSR, lipoamide dehydrogenase, and mercuric reductase. Most of these enzymes are homodimeric flavoproteins of M_r ~ 100 kD that utilize a conserved active-site disulfide bond to effect catalysis.

Antimicrobial agents commonly used to combat microorganism infections generally interfere with a critical step in the metabolism of the

microorganism resulting in growth inhibition or death thereof. However, pathogenic microorganisms, including staphylococci and enterococci, are developing resistance, and in many cases multiple resistances, to existing antimicrobial agents.

S. aureus is an opportunistic pathogen of increasing medical concern. It can be aggressively invasive, spreading rapidly through soft tissues, directly invading bones and even entering the bloodstream in which it may produce septic shock and disseminated intravascular coagulation. Two categories of disease may be ascribed to staphylococci: those related to toxins produced by the organism (*S. aureus* exclusively), including gastroenteritis, toxic shock syndrome, scalded skin syndrome, and the like; and those related to direct invasion and systemic spread of the organism, including dermal infections, bone and joint infections, staphylococcal pneumonia and empyema, meningitis, cerebritis, endocarditis, bacteremia, septic shock, and the like.

Strains of β-lactam antibiotic resistant staphylococci (BLARS), otherwise referred to as methicillin-resistant *S. aureus* (MRSA), have become a widespread cause of fatal nosocomial infection. Infections caused by such resistant staphylococci are treated predominantly by the "last resort" antibiotic, vancomycin. Newer antimicrobial agents that may be effective against staphylococcal infections include the investigational agent teichoplanin and the quinolones; however, recent data indicate increasing quinolone resistance. Since vancomycin resistance would essentially exhaust the current antibiotic therapeutic arsenal, it is now mandatory to identify new cellular targets and new chemotherapeutic agents effective against MRSA.

Accordingly, there is a need for new antimicrobial agents to which microorganisms are susceptible. The ability to discover and use such agents would be augmented by the availability of new cellular targets. Acquired resistance that protects against or compensates for disruption of one metabolic pathway by a particular class of antimicrobial agents would be unlikely to have a similarly protective or compensatory effect for disruption of a distinct metabolic pathway.

Summary of the Invention

The inventors herein have identified an enzyme that catalyzes the specific NADPH-dependent reduction of CoA disulfide. The enzyme, Coenzyme A disulfide reductase (CoADR), catalyzes the specific reduction of Coenzyme A disulfide to Coenzyme A with the concomitant oxidation of NADPH to $NADP^+$ as shown below:

$$CoASSCoA + NADPH + H^+ \xrightarrow{CoADR} 2\ CoASH + NADP^+.$$

This is a significant metabolic function in staphylococci, some enterococci, other Gram-positive bacteria, and other microorganisms that do not produce glutathione (GSH) but instead rely on Coenzyme A as the predominant cellular redox buffer. Inhibition of CoADR causes CoA disulfide to build up and depletes the pool of CoA that is available to act as a cofactor in numerous metabolic processes, including acyl transfer reactions, fatty acid biosynthesis, radical scavenging, peroxidase reactions, S-transferase drug resistance, other disulfide reductase reactions, disulfide isomerase reactions, and ribonucleotide reductase reactions. Such compromised cells are thus more likely to succumb to environmental challenges, such as those posed by a host immune system. As a result, inhibitors of CoADR activity are effective antimicrobial agents against *S. aureus* and other microorganisms that depend on CoA as a redox buffer. In addition, GSR need not be affected by specific inhibitors of CoADR. Thus, inhibition of microorganisms may be effected by inhibiting CoADR with few or no side effects in a eukaryotic host organism.

Accordingly, in one embodiment, the invention is directed to an isolated *S. aureus* CoADR polypeptide.

In another embodiment, the invention is directed to a DNA molecule that encodes an *S. aureus* CoADR polypeptide.

In yet another embodiment, the invention is directed to a recombinant vector comprising such a DNA molecule.

In still other embodiments, the invention is directed to messenger RNA encoded by the DNA, recombinant host cells transformed with vectors comprising the DNA and methods of producing recombinant polypeptides using the transformed cells.

In still another embodiment of the invention, antibodies to the *S. aureus* CoADR polypeptide are provided.

In a further embodiment, the invention is directed to a method of identifying compounds that modulate CoADR activity.

In still a further embodiment, the invention is directed to a method for inhibiting the growth of microorganisms that utilize Coenzyme A as their predominant redox buffer.

In yet a further embodiment, the invention is directed to a method of treating an individual infected with a Gram-positive bacteria by administering a therapeutically effective amount of a CoADR activity-modulating compound.

In yet another embodiment, the invention is directed to a class of antimicrobial agents.

In still a further embodiment, the invention is directed to a method of detecting the presence of *S. aureus* in a test sample.

In yet a further embodiment, the invention is directed to diagnostic kits comprising (a) an oligomer probe for detecting the presence of polynucleotides that encode *S. aureus* CoADR, (b) an antibody capable of specifically binding to the CoADR polypeptide for detecting the presence and/or amount of *S. aureus* CoADR in a test sample, as well as for detecting of the presence of *S. aureus*, and (c) an *S. aureus* CoADR polypeptide for screening compounds for CoADR-modulating activity or for screening test samples for the presence of a CoADR-antibody containing.

These and other embodiments of the present invention will readily occur to those of ordinary skill in the art in view of the disclosure herein.

Brief Description of the Figures

FIG. 1 shows the nucleotide sequence (SEQ ID NO:___) of the *S. aureus* open reading frame encoding CoADR.

FIG. 2 shows the deduced amino acid sequence (SEQ ID NO:___) of the *S. aureus* CoADR derived from the nucleotide sequence of the open reading frame.

Detailed Description

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology and recombinant DNA technology, which are within the skill of the art. Such techniques are explained fully in the literature. *See*, e.g., Sambrook, Fritsch & Maniatis, *Molecular Cloning: A Laboratory Manual*, Second Edition (1989); *DNA Cloning*, Vols. I and II (D.N. Glover ed. 1985); Perbal, B., *A Practical Guide to Molecular Cloning* (1984); the series, *Methods In Enzymology* (S. Colowick and N. Kaplan eds., Academic Press, Inc.); *Transcription and Translation* (Hames et al. eds. 1984); *Gene Transfer Vectors For Mammalian Cells* (J. H. Miller et al. eds. (1987) Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.); Scopes, *Protein Purification: Principles and Practice* (2nd ed., Springer-Verlag); and *PCR: A Practical Approach* (McPherson et al. eds. (1991) IRL Press).

All patents, patent applications and publications cited herein, whether *supra* or *infra*, are hereby incorporated by reference in their entirety. As used in this specification and the appended claims, the singular forms "a," "an" and "the" include plural references unless the content clearly dictates otherwise.

A. Definitions

In describing the present invention, the following terms will be employed, and are intended to be defined as indicated below.

The term "polynucleotide" as used herein means a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides. This term refers only to the primary structure of the molecule. Thus, the term includes double- and single-stranded DNA, as well as double- and single-stranded RNA. It also includes modifications, such as by methylation and/or by capping, and unmodified forms of the polynucleotide.

"Polypeptide" and "protein" are used interchangeably herein and indicate a molecular chain of amino acids linked through peptide bonds. The terms do not refer to a specific length of the product. Thus, peptides, oligopeptides, and proteins are included within the definition of polypeptide. The terms include post-translation modifications of the polypeptide, for example, glycosylations, acetylations, phosphorylations, and the like. In addition, protein fragments, analogs, muteins, fusion proteins and the like are included within the meaning of polypeptide. Thus, by "CoADR polypeptide" is meant a polypeptide, whether isolated, recombinant or synthetic, comprising an amino acid sequence identical to that depicted in Fig. 2, and fragments thereof that include only so much of the molecule as necessary for the polypeptide to retain biological activity, e.g., catalytic and/or immunological activity, as well as analogs, mutated or variant proteins, and the like, thereof that retain such activity.

"Recombinant host cells," "host cells," "cells," "cell lines," "cell cultures," and other such terms denoting microorganisms or higher eukaryotic cell lines cultured as unicellular entities refer to cells which can be, or have been, used as recipients for recombinant vectors or other transfer DNA, immaterial of the method by which the DNA is introduced into the cell or the subsequent disposition of the cell. The terms include the progeny of the original cell which has been transfected.

A "vector" is a replicon in which another polynucleotide segment is attached, such as to bring about the replication and/or expression

of the attached segment. The term includes expression vectors, cloning vectors, and the like.

The term "control sequence" refers to a polynucleotide sequence which effects the expression of coding sequences to which it is ligated. The nature of such a control sequence differs depending upon the host organism. In prokaryotes, such control sequences generally include a promoter, a ribosomal binding site, and a terminator. In eukaryotes, such control sequences generally include a promoter, a terminator and, in some instances, an enhancer. The term "control sequence" thus is intended to include at a minimum all components necessary for expression, and also may include additional components whose presence is advantageous, for example, leader sequences.

A "coding sequence" is a polynucleotide sequence that is transcribed into mRNA and/or translated into a polypeptide when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a translation start codon at the 5'-terminus and a translation stop codon at the 3'-terminus. A coding sequence can include, but is not limited to, mRNA, cDNA, and recombinant polynucleotide sequences. Mutants or analogs may be prepared by the deletion of a portion of the coding sequence, by insertion of a sequence, and/or by substitution of one or more nucleotides within the sequence. Techniques for modifying nucleotide sequences, such as site-directed mutagenesis, are well known to those skilled in the art. *See, e.g.*, Sambrook et al., *supra*; *DNA Cloning*, Vols. I and II, *supra*; *Nucleic Acid Hybridization*, *supra*.

"Operably linked" refers to a situation wherein the components described are in a relationship permitting them to function in their intended manner. Thus, for example, a control sequence "operably linked" to a coding sequence is ligated in such a manner that expression of the coding sequence is achieved under conditions compatible with the control sequences. A coding sequence may be operably linked to control sequences that direct the

transcription of the polynucleotide whereby said polynucleotide is expressed in a host cell

The term "transfection" refers to the insertion of an exogenous polynucleotide into a host cell, irrespective of the method used for the insertion, or the molecular form of the polynucleotide that is inserted. For example, injection, direct uptake, transduction, and f-mating are included. Furthermore, the insertion of a polynucleotide *per se* and the insertion of a plasmid or vector comprised of the exogenous polynucleotide are also included. The exogenous polynucleotide may be directly transcribed and translated by the cell, maintained as a nonintegrated vector, for example, a plasmid, or alternatively, may be stably integrated into the host genome.

By the term "degenerate variant" or "structurally conserved mutation" is intended a polynucleotide containing changes in the nucleic acid sequence thereof, such as insertions, deletions or substitutions, that encodes a polypeptide having the same amino acid sequence as the polypeptide encoded by the polynucleotide from which the degenerate variant is derived.

The term "isolated," when referring to a polynucleotide or a polypeptide, intends that the indicated molecule is present in the substantial absence of other similar biological macromolecules of the same type. The term "isolated" as used herein means that at least 75 wt.%, more preferably at least 85 wt.%, more preferably still at least 95 wt.%, and most preferably at least 98 wt.% of a composition is the isolated polynucleotide or polypeptide. An "isolated polynucleotide" that encodes a particular polypeptide refers to a polynucleotide that is substantially free of other nucleic acid molecules that do not encode the subject polypeptide; however, the molecule may include conservative mutations as defined herein.

The term "test sample" refers to a component of an individual's body which is the source of an analyte, such as antibodies or antigens of interest. These test samples include biological samples which can be tested by the methods of the present invention described herein and include human and animal body fluids such as whole blood, serum, plasma, cerebrospinal fluid,

urine, lymph fluids, and various external secretions of the respiratory, intestinal and genitorurinary tracts, tears, saliva, milk, white blood cells, myelomas and the like; biological fluids such as cell culture supernatants; fixed tissue specimens; and fixed cell specimens.

The following single-letter amino acid abbreviations are used throughout the text:

Alanine	A	Arginine	R
Asparagine	N	Aspartic acid	D
Cysteine	C	Glutamine	Q
Glutamic acid	E	Glycine	G
Histidine	H	Isoleucine	I
Leucine	L	Lysine	K
Methionine	M	Phenylalanine	F
Proline	P	Serine	S
Threonine	T	Tryptophan	W
Tyrosine	Y	Valine	V

B. General Methods

The present invention is based on the identification of an *S. aureus* CoADR, as well as a polynucleotide encoding the CoADR, and methods of making the CoADR. The invention includes not only the enzyme but also methods for screening compounds for pharmacological activity using the enzyme, cells expressing the enzyme, monoclonal antibodies to the enzyme and the use of the antibodies to diagnose microbial infections/disease.

In particular, the inventors herein have identified a new CoADR enzyme in *S. aureus*. CoADR has been purified to homogeneity therefrom. The native enzyme has a molecular mass (M_r) of approximately 100 kD as determined by gel exclusion chromatography. The enzyme exists as a homodimer having a subunit M_r of about 50 kD and binding one flavin adenine nucleotide (FAD) per subunit.

The enzyme can be isolated directly from bacteria as follows. Bacteria can be cultured in a suitable culture medium, such as trypticase soy broth (TSB). The bacteria are then removed from the culture medium using standard techniques known in the art, such as by centrifugation or microfiltration or a combination of the two. For example, microfiltration using an appropriate filter will suffice to remove unwanted cellular debris.

Bacteria thus obtained are prepared to release the contents of the cytoplasm. Bacterial cells may be broken using methods and/or reagents known in the art that do not adversely affect the structure and/or the activity of the CoADR, e.g., exposure to freeze-thaw cycles, exposure to an ultrasonic disintegrator, homogenization, bead milling, chemical or enzymatic cell lysis, and the like. In one preferred method, cells are incubated in a buffer containing lysostaphin, a lytic agent for *S. aureus*, and then passed through a French pressure cell.

The bacterial cell medium thus prepared can be further processed to separate the protein from the cellular debris, and provide an initial stage of purification and volume reduction. For example, the lysate obtained from the previous step may be processed by a primary separation procedure such as ultrafiltration, that is passage through a filter having a particular weight cut-off, to concentrate the sample by reducing the water and salts content. Alternatively, the lysate may be precipitated by neutral salts such as ammonium sulphate, organic solvents such as ethanol, or other agents for recovering and purifying the protein. Preferably, *S. aureus* CoADR is precipitated from the lysate by adding ammonium sulfate to the lysate to approximately 40%, preferably 50%, saturation. The supernatant of the same is collected by, e.g., centrifugation, and the ammonium sulfate is adjusted to 90%, preferably 80%, saturation. The treated precipitate thus obtained is collected and used in further purification steps.

A number of protein purification operations may be used to further purify the *S. aureus* CoADR including adsorption chromatography, ion-exchange chromatography, hydrophobic interaction chromatography,

affinity chromatography, chromatofocussing, gel filtration, reversed-phase liquid chromatography, phosphocellulose chromatography, hydroxyapatite chromatography or lectin chromatography, any combination of such techniques. Protein refolding steps can be used, as necessary, in completing configuration of the protein. Finally, high performance liquid chromatography (HPLC) can be employed for final purification steps.

Preferably, a first purification step will be one of high resolution in order to minimize the number of stages used and hence maximize yield. More preferably, the first purification step is an affinity purification using, e.g., 2',5'-ADP linked to an appropriate support matrix as the affinity adsorbent. The affinity purification may be done in a batch mode, by which the sample is adsorbed onto the affinity matrix and eluted in a single step, by progressive elution without a change in the elution buffer or by a gradient elution, in which the buffer is continuously changed to effect elution of the enzyme. Preferably, the CoADR is eluted from the affinity matrix with a linear salt gradient.

A subsequent purification step may also be used to "polish" the preparation obtained from the affinity purification step. Preferably, the subsequent step is an ion-exchange purification step, more preferably an anion-exchange purification step. Suitable anion exchangers include a wide variety of materials, known in the art. Particularly preferred are strong anion exchangers capable of binding CoADR over a wide pH range. For example, quaternary ammonium and quaternary alkylalkanolammonium anion exchange matrices are particularly useful for use herein. Useful matrix materials include but are not limited to, cellulose matrices, such as fibrous, microgranular and beaded matrices; agarose, dextran, polyacrylate, polyvinyl, polystyrene, silica and polyether matrices; and composites. Particularly preferred herein are matrices containing the functional ligand $R\text{-}NH_4^+$, preferably sulfopropyl resins. Representative matrices include MonoQ HR 5/5 or SigmaChrom IEX-Q.

Once purified, the amino acid sequences of the proteins can be determined, e.g., by repetitive cycles of Edman degradation, followed by amino acid analysis by HPLC. Other methods of amino acid sequencing are also known in the art. Using such techniques, the N-terminal fourteen amino acids of the purified *S. aureus* CoADR polypeptide has been determined to be

Pro-Lys-Ile-Val-Val-Val-Gly-Ala-Val-Ala-Gly-Gly-Ala-Thr

(SEQ ID NO:____). The complete deduced amino acid sequence is shown in FIG. 2.

Based on knowledge of the amino acid sequence, DNA encoding the enzyme can be derived from genomic or cDNA, prepared by synthesis, or by a combination of techniques. The DNA can then be used to express the CoADR or as a template for the preparation of RNA using methods well known in the art (*see*, Sambrook et al., *supra*).

More particularly, DNA encoding the *S. aureus* CoADR may be obtained from an appropriate DNA library, e.g., an *S. aureus* genomic DNA library. DNA libraries may be probed using the procedure described by Grunstein et al. (1975) *Proc. Natl. Acad. Sci. USA* 73:3961. Briefly, the DNA to be probed is immobilized on nitrocellulose filters, denatured and prehybridized with a buffer which contains 0-50% formamide, 0.75 M NaCl, 75 mM Na citrate, 0.02% (w/v) each of bovine serum albumin (BSA), polyvinyl pyrollidone and Ficoll®, 50 mM Na phosphate (pH 6.5), 0.1% sodium dodecyl sulfate (SDS) and 100 μg/ml carrier denatured DNA. The percentage of formamide in the buffer, as well as the time and temperature conditions of the prehybridization and subsequent hybridization steps, depends on the stringency required. Oligomeric probes which require lower stringency conditions are generally used with low percentages of formamide, lower temperatures, and longer hybridization times. Probes containing more than 30 or 40 nucleotides such as those derived from cDNA or genomic sequences generally employ higher temperatures, for example, about 40°C to 42°C, and a high percentage, for example, 50% formamide. Following prehybridization, a ^{32}P-labelled oligonucleotide probe is added to the buffer, and the filters are

incubated in this mixture under hybridization conditions. After washing, the treated filters are subjected to autoradiography to show the location of the hybridized probe. DNA in corresponding locations on the original agar plates is used as the source of the desired DNA.

Synthetic oligonucleotides may be prepared using an automated oligonucleotide synthesizer such as that described by Warner (1984) *DNA* 3:401. If desired, the synthetic strands may be labelled with ^{32}P by treatment with polynucleotide kinase in the presence of ^{32}P-ATP, using standard conditions for the reaction. DNA sequences including those isolated from genomic or cDNA libraries, may be modified by known methods which include site-directed mutagenesis as described by Zoller (1982) *Nucleic Acids Res.* 10:6487. Briefly, the DNA to be modified is packaged into phage as a single stranded sequence, and converted to a double stranded DNA with DNA polymerase using, as a primer, a synthetic oligonucleotide complementary to the portion of the DNA to be modified, and having the desired modification included in its own sequence. Culture of the transformed bacteria, which contain replications of each strand of the phage, are plated in agar to obtain plaques. Theoretically, 50% of the new plaques contain phage having the mutated sequence, and the remaining 50% have the original sequence. Replicates of the plaques are hybridized to labelled synthetic probe at temperatures and conditions suitable for hybridization with the correct strand, but not with the unmodified sequence. The sequences which have been identified by hybridization are recovered and cloned.

Once produced, the DNA may then be incorporated into a cloning vector or an expression vector for replication in a suitable host cell. Vector construction employs methods known in the art. Generally, site-specific DNA cleavage is performed by treating with suitable restriction enzymes under conditions which generally are specified by the manufacturer of these commercially available enzymes. Usually, about 1 microgram (μg) of plasmid or DNA sequence is cleaved by 1-10 units of enzyme in about 20 μl of buffer solution by incubation at 37°C for 1 to 2 hours. After incubation

with the restriction enzyme, protein is removed by phenol/chloroform extraction and the DNA recovered by precipitation with ethanol. The cleaved fragments may be separated using polyacrylamide or agarose gel electrophoresis methods, according to methods known by those of skill in the art.

Sticky end cleavage fragments may be blunt ended using *E. coli* DNA polymerase 1 (Klenow) in the presence of the appropriate deoxynucleotide triphosphates (dNTPs) present in the mixture. Treatment with S1 nuclease also may be used, resulting in the hydrolysis of any single stranded DNA portions.

Ligations are performed using standard buffer and temperature conditions using T4 DNA ligase and ATP. Sticky-end ligations require less ATP and less ligase than blunt-end ligations. When vector fragments are used as part of a ligation mixture, the vector fragment often is treated with bacterial alkaline phosphatase (BAP) or calf intestinal alkaline phosphatase to remove the 5'-phosphate and thus prevent religation of the vector. Alternatively, restriction enzyme digestion of unwanted fragments can be used to prevent ligation.

For standard vector constructions, ligation mixtures are transformed into a suitable host, and successful transformants selected by antibiotic resistance or other markers. Plasmids from the transformants can then be prepared according to the method of Clewell et al. (1969) *Proc. Natl. Acad. Sci. USA* 62:1159 usually following chloramphenicol amplification as reported by Clewell et al. (1972) *J. Bacteriol.* 110:667. The DNA is isolated and analyzed usually by restriction enzyme analysis and/or sequencing. Sequencing may be by the well-known dideoxy method of Sanger et al. (1977) *Proc. Natl. Acad. Sci. USA* 74:5463) as further described by Messing et al. (1981) *Nucleic Acid Res.* 9:309, or by the method reported by Maxam et al. (1980) *Meth. Enzymol.* 65:499. Problems with band compression, which are sometimes observed in GC rich regions, are overcome by use of

T-deazoguanosine according to the method reported by Barr et al. (1986) *Biotechniques* 4:428.

Host cells are genetically engineered (transduced or transformed or transfected) with the vectors of this invention which may be a cloning vector or an expression vector. The vector may be in the form of a plasmid, a viral particle, a phage, etc. The engineered host cells can be cultured in conventional nutrient media modified as appropriate for activating promoters, selecting transformants or amplifying the CoADR-encoding polynucleotide. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, and will be apparent to those of skill in the art.

Both prokaryotic and eukaryotic host cells may be used for expression of desired coding sequences when appropriate control sequences that are compatible with the designated host are used. Among prokaryotic hosts, *Escherichia coli* is frequently used. Expression control sequences for prokaryotes include promoters, optionally containing operator portions, and ribosome binding sites. Transfer vectors compatible with prokaryotic hosts are commonly derived from the plasmid pBR322 that contains operons conferring ampicillin and tetracycline resistance, and the various pUC vectors, that also contain sequences conferring antibiotic resistance markers. These markers may be used to obtain successful transformants by selection. Commonly used prokaryotic control sequences include the beta-lactamase (penicillinase), lactose promoter system (Chang et al. (1977) *Nature* 198:1056), the tryptophan promoter system (reported by Goeddel et al. (1980) *Nucleic Acid Res.* 8:4057) and the lambda-derived Pl promoter and N gene ribosome binding site (Shimatake et al. (1981) *Nature* 292:128) and the hybrid *Tac* promoter (De Boer et al. (1983) *Proc. Natl. Acad. Sci. USA* 292:128) derived from sequences of the *trp* and *lac* UV5 promoters. The foregoing systems are particularly compatible with *E. coli*; however, other prokaryotic hosts such as strains of *Bacillus* or *Pseudomonas* may be used if desired, with corresponding control sequences.

Eukaryotic hosts include yeast and mammalian cells in culture systems. *Saccharomyces cerevisiae* and *S. carlsbergensis* are commonly used yeast hosts, and are convenient fungal hosts. Yeast-compatible vectors carry markers that permit selection of successful transformants by conferring protrophy to auxotrophic mutants or resistance to heavy metals on wild-type strains. Yeast compatible vectors may employ the 2-micron origin of replication (Broach et al. (1983) *Meth. Enzymol.* 101:307), the combination of CEN3 and ARS1 or other means for assuring replication, such as sequences that will result in incorporation of an appropriate fragment into the host cell genome. Control sequences for yeast vectors are known in the art and include promoters for the synthesis of glycolytic enzymes, including the promoter for 3-phosphoglycerate kinase. *See*, for example, Hess et al. (1968) *J. Adv. Enzyme Reg.* 7:149, Holland et al. (1978) *Biochemistry* 17:4900, and Hitzeman (1980) *J. Biol. Chem.* 255:2073. Terminators also may be included, such as those derived from the enolase gene as reported by Holland (1981) *J. Biol. Chem.* 256:1385. It is contemplated that particularly useful control systems are those that comprise the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter or alcohol dehydrogenase (ADH) regulatable promoter, terminators also derived from GAPDH, and, if secretion is desired, leader sequences from yeast alpha factor. In addition, the transcriptional regulatory region and the transcriptional initiation region which are operably linked may be such that they are not naturally associated in the wild-type organism.

Mammalian cell lines available as hosts for expression are known in the art and include many immortalized cell lines which are available from the American Type Culture Collection. These include HeLa cells, Chinese hamster ovary (CHO) cells, baby hamster kidney (BHK) cells, and others. Suitable promoters for mammalian cells also are known in the art and include viral promoters such as that from Simian Virus 40 (SV40), Rous sarcoma virus (RSV), adenovirus (ADV), bovine papilloma virus (BPV), cytomegalovirus (CMV). Mammalian cells also may require terminator

sequences and poly A addition sequences; enhancer sequences which increase expression also may be included, and sequences which cause amplification of the gene also may be desirable. These sequences are known in the art. Vectors suitable for replication in mammalian cells may include viral replicons, or sequences which insure integration of the appropriate sequences encoding the *S. aureus* CoADR into the host genome.

Other eukaryotic systems are also known, as are methods for introducing polynucleotides into such systems, such as into amphibian cells, using known methods, and insect cells using methods described in Summers and Smith (1987), Texas Agricultural Experiment Station Bulletin No. 1555 (1987), and the like.

Transformation may be by any known method for introducing polynucleotides into a host cell, including packaging the polynucleotide in a virus and transducing a host cell with the virus, by direct uptake of the polynucleotide by the host cell, and the like. The transformation procedures selected depend upon the host to be transformed. Bacterial transformation by direct uptake generally employs treatment with calcium or rubidium chloride. Cohen (1972) *Proc. Natl. Acad. Sci. USA* 69:2110. Yeast transformation by direct uptake may be conducted using the calcium phosphate precipitation method of Graham et al. (1978) *Virology* 52:526, or modification thereof.

Expression of active CoADR can be assayed colorimetrically by monitoring the NADPH- and CoA disulfide-dependent reduction of 5,5'-dithio-*bis*-2-nitrobenzoic acid (DTNB) at 412 nm. This reaction is suitable for screening compounds for their CoADR-inhibiting activity. Alternatively, expression of CoADR can be monitored using an ELISA assay and antibodies prepared to the isolated CoADR enzyme. The enzyme is recovered and purified from recombinant host cell cultures expressing the same by known methods as described above.

The *S. aureus* CoADR polypeptide, or fragments thereof, of the present invention may also be synthesized by conventional techniques known in the art, for example, by chemical synthesis such as solid phase peptide

synthesis. Such methods are known to those skilled in the art. In general, these methods employ either solid or solution phase synthesis methods, well known in the art. *See, e.g.*, J. M. Stewart and J. D. Young, *Solid Phase Peptide Synthesis*, 2nd Ed., Pierce Chemical Co., Rockford, IL (1984) and G. Barany and R. B. Merrifield, *The Peptides: Analysis, Synthesis, Biology*, editors E. Gross and J. Meienhofer, Vol. 2, Academic Press, New York, (1980), pp. 3-254, for solid phase peptide synthesis techniques; and M. Bodansky, *Principles of Peptide Synthesis*, Springer-Verlag, Berlin (1984) and E. Gross and J. Meienhofer, Eds., *The Peptides: Analysis, Synthesis, Biology*, *supra*, Vol. 1, for classical solution synthesis.

Once obtained, the enzyme may be used to identify compounds that modulate *S. aureus* CoADR activity. Thus, as described above, enzyme activity and the effects of compounds on enzyme activity can be assayed colorimetrically by monitoring the NADPH- and CoA disulfide-dependent reduction of 5,5'-dithio-*bis*-2-nitrobenzoic acid (DTNB) at 412 nm. Using this assay method, it has been determined that pantethine derivatives are effective as inhibitors of CoADR activity. Purification or expression of *S. aureus* CoADR and screening for compounds that inhibit the enzyme activity provides a method for rapid selection of compounds with enzyme-inhibiting activity.

Accordingly, compounds that inhibit *S. aureus* CoADR are considered potential therapeutic agents for use in treating several disorders including, without limitation, staphylococcal, enterococcal and other Gram-positive bacterial infections, and the like, in which such agents may be useful in preventing growth and/or reproduction of the infecting microorganism when administered in a suitable pharmaceutical composition. Examples of such diseases for which CoADR inhibitors are useful therapeutic agents include, gastroenteritis, toxic shock syndrome, scalded skin syndrome, dermal infections, bone and joint infections, pneumonia and empyema, meningitis, cerebritis, endocarditis, bacteremia, septic shock, septicemia, food poisoning, enteritis, and the like.

The inhibitory compounds of the present invention can be formulated into therapeutic compositions in a variety of dosage forms such as, but not limited to, liquid solutions or suspensions, tablets, pills, powders, ointments suppositories, polymeric microcapsules or microvesicles, liposomes, and injectable or infusible solutions. The preferred form depends upon the mode of administration and the particular microorganism and disease type targeted. The compositions also preferably include pharmaceutically acceptable vehicles, carriers or adjuvants, well known in the art, such as human serum albumin, ion exchangers, alumina, lecithin, buffer substances such as phosphates, glycine, sorbic acid, potassium sorbate, and salts or electrolytes such as protamine sulfate. Suitable vehicles are, for example, water, saline, dextrose, glycerol, ethanol, or the like, and combinations thereof. Actual methods of preparing such compositions are known, or will be apparent, to those skilled in the art. *See, e.g., Remington's Pharmaceutical Sciences*, Mack Publishing Company, Easton, Pennsylvania, 18th edition, 1990.

The above compositions can be administered using conventional modes of delivery including, but not limited to, intravenous, intraperitoneal, oral, intralymphatic, or subcutaneous administration. Local administration to a tissue in question, or to a site of infection, e.g., direct injection into an infected joint, will also find use with the present invention.

Therapeutically effective doses will be easily determined by one of skill in the art and will depend on the severity and course of the disease, the patient's health and response to treatment, and the judgment of the treating physician.

Furthermore, the *S. aureus* CoADR polypeptide can be used to prepare polyclonal or monoclonal antibodies using techniques that are well known in the art. The *S. aureus* CoADR can be purified from a culture of *S. aureus* or can be obtained using the recombinant technology outlined below, i.e., a recombinant cell that expresses the enzyme can be cultured to produce quantities of the enzyme that can be recovered and isolated. Alternatively, the

enzyme can be synthesized using conventional polypeptide synthetic techniques as provided below. Monoclonal antibodies that display specificity and selectivity for the enzyme can be labeled with a detectable moiety, e.g., a fluorescent moiety, and used in *in vitro,* or *in situ* immunofluorescent assays, or the like. The antibodies can be used to identify *S. aureus* for immunodiagnostic purposes.

In addition, DNA encoding the *S. aureus* CoADR, or RNA derived therefrom, can be used to design oligonucleotide probes for *S. aureus* present in a host organism. As used herein, the term "probe" refers to a structure comprised of a polynucleotide, as defined above, which contains a nucleic acid sequence complementary to a nucleic acid sequence present in a target polynucleotide. The polynucleotide regions of probes may be composed of DNA, and/or RNA, and/or synthetic nucleotide analogs such as morpholino compounds and peptide nucleic acid ("PNA") analogs. Such probes may be used in *in vitro* or *in situ* hybridization assays, or the like, and are useful, for example, for the diagnosis of microbial infections.

Using a determined portion of the isolated CoADR-encoding polynucleotide, oligomers of approximately eight or more nucleotides can be prepared, either by excision or synthetically, which hybridize with the CoADR-encoding polynucleotide. Such oligomers are useful, for example, for detecting the presence of bacteria in diseased individuals. The natural or derived probes for CoADR polynucleotides are a length that allows the detection of unique sequences by hybridization. While six to eight nucleotides may be a workable length, sequences of ten to twelve nucleotides are preferred, and those of about twenty nucleotides most preferred. These probes can be prepared using routine, standard methods including automated oligonucleotide synthetic methods.

When the oligonucleotide probes are to be used as diagnostic reagents, the test sample to be analyzed, such as blood or serum, may be treated such as to extract a nucleic acid fraction thereof. The resulting nucleic acid from the sample may be subjected to gel electrophoresis or other size

separation techniques, or the nucleic acid sample may be dot-blotted without size separation. The sample is then exposed to an oligonucleotide probe that has been detectably labelled. Suitable labels and methods for attaching labels to probes are known in the art, and include but are not limited to radioactive labels incorporated by nick translation or kinasing, biotin, fluorescent and chemiluminescent probes, enzymes which catalyze the production of a detectable product such as horseradish peroxidase, alkaline phosphatase, β-galactosidase, and the like. The nucleic acids extracted from the sample are then treated with the labelled probe under conditions of suitable hybridization stringency.

The stringency of hybridization is determined by a number of factors during the washing procedure, including temperature, ionic strength, length of time and concentration of formamide. Sambrook et al., *supra*. Hybridization can be carried out by a number of various techniques. Amplification of the sample nucleic acid, if required, can be performed, for example, by ligase chain reaction (LCR), polymerase chain reaction (PCR), Q-beta replicase, NASBA, or other techniques well known in the art. The amplified nucleic acids then may be detected using a hybridization assay such as those known in the art.

CoADR, antibodies thereto, as well as polynucleotides encoding CoADR or portions thereof, can be provided in diagnostic kits. For example, oligomer probes capable of specifically hybridizing to a polynucleotide encoding a CoADR can be packaged in diagnostic kits which include the probe nucleic acid sequence which may be labelled. Alternatively, the probe may be provided unlabelled and the ingredients for labelling could be included with the kit. The kit also may contain other suitably packaged reagents and materials needed or desirable for the particular hybridization protocol, for example, standards as well as instructions for performing the assay.

In addition, kits can include reagents for detecting of the presence and/or amount of *S. aureus* CoADR in a test sample, as well as for

detecting of the presence of *S. aureus*. Such reagents can comprise, e.g., an antibody capable of specifically binding to the CoADR polypeptide.

Furthermore, kits containing an *S. aureus* CoADR polypeptide in a suitable container are provided for screening compounds for CoADR-modulating activity or for screening test samples for the presence of a CoADR-antibody. It is contemplated that reagents employed in the above kits can be provided in one or more containers such as vials or bottles, with each container containing a separate reagent such as a monoclonal antibody, or a cocktail of monoclonal antibodies, or a polypeptide (either recombinant or synthetic) employed in the assay. Other components such as buffers, controls, and the like, known to those of ordinary skill in art, may be included in such test kits. The kits will also include instructions for the use thereof.

Below are examples of specific embodiments for carrying out the present invention. The examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

Efforts have been made to ensure accuracy with respect to numbers used (*e.g.*, amounts, temperatures, etc.), but some experimental error and deviation should, of course, be allowed for.

Experimental

STRAINS AND MEDIA. *Staphylococcus aureus* R8325-4 (no prophage), used as a source of CoADR and genomic DNA, was obtained from John Iandolo, Kansas State University, Department of Pathobiology (Manhattan, KS). *S.aureus* was grown in tryptic soy broth (Difco Laboratories, Detroit, MI) at 30°C under standard incubation conditions. *Escherichia coli* DH5a was from Gibco, BRL, strain BL21 (DE3) and plasmid pET22B(+) were from Novagen (Madison, WI). *E. coli* was grown in LB and TB medium at 37°C. When required, *E. coli* was grown in the presence of ampicillin (100-400 mg/mL).

MATERIALS. Reduced nicotinamide adenine dinucleotide phosphate (NADPH), riboflavin, flavin adenine mononucleotide (FMN), flavin adenine dinucleotide (FAD), coenzyme A disulfide, glutathionyl-coenzyme A mixed disulfide, 3'-dephospho coenzyme A, 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), lysostaphin, bicinchoninic acid solution, 4% copper sulphate pentahydrate solution, 2',5'-adenosine diphosphate (ADP)-Sepharose®; and phenylmethylsulphonyl fluoride (PMSF) were from Sigma (Mississauga, ON). 3'-dephospho CoA was oxidized to the disulfide by incubation at room temperature overnight in Tris-HCl (20 mM), pH 9.0, containing copper (5 μM). 4,4'-phosphopantetheine was formed by incubation of CoA with nucleotide pyrophosphatase. The thiol was oxidized as described above, and the disulfide was purified by high performance liquid chromatography (HPLC). All other chemicals were of reagent grade or better and were used without further purification.

GENERAL METHODS. Isolation of *S. aureus* genomic DNA was carried out by standard methods, Novick (1991) *Meth. Enzymol.* 204:587-636. Oligonucleotides were prepared on a Beckman oligonucleotide synthesizer using standard phosphoramidite chemistry. Restriction enzymes and Taq DNA polymerase were from Gibco BRL, and T4 DNA ligase and calf intestine alkaline phosphatase were from New England Biolabs. DNA fragments and PCR products were routinely purified using Qiaquick spin columns (Qiagen, San Diego, CA). DNA fragments were labeled with digoxigenin by random primed PCR using the DIG DNA Labeling and Detection Kit (Boehringer Mannheim, Laval, Québec). Plasmids were purified on Qiawell cartridges (Qiagen) and sequenced using the Dye Termination Cycle Sequencing Kit and AmpliTaq DNA polymerase, FS (Perkin Elmer) and analyzed on an ABI 373 automated DNA sequence analyzer. CoADR was purified as described above. All other reagents were of standard grade and used without further purification.

Protein chromatography was performed on a Fast Phase Liquid Chromatography (FPLC) system (Pharmacia, Upsala) equipped with UV and conductivity flow cells. Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and blotting of proteins were carried out using a Mini Protean Electrophoresis system (Bio-Rad, Richmond, CA) using a Tris-glycine buffer. Prestained protein standards for SDS-PAGE were from GibcoBRL and were 200, 97, 68, 43, 29, 18, and 14 kD. Molecular weight standards for gel filtration were from BioRad and were 670, 158, 44, 17, and 1.3 kD. Spectrophotometric measurements were performed on a thermal-stated Cary I spectrophotometer (Varian, Australia) using quartz cuvettes (500 ml) (Hellma, Concord, ON). Concentration of protein samples were carried out using centricon filters (Amicon). Dialysis tubing was from Spectrum Medical Industries, Inc.

Concentrations of FAD, NADPH and CoA disulfide (and dephosphoCoA disulfide) were measured spectrophotometrically at 340 nm (e^{340} = 6220 $M^{-1}cm^{-1}$), 260 nm (e^{260} = 33,600 $M^{-1}cm^{-1}$), and 450 nm (e^{450}= 11,000 $M^{-1}cm^{-1}$), respectively. DTNB assays were performed in Tris-HCl (20 mM), pH 8.0, containing EDTA (1 mM) (TE buffer), and were monitored at 412 nm for the nitrobenzothiolate anion (e^{412} = 15,600 M^{-1} cm^{-1} (Ellman (1959) *Arch. Biochem. Biophys.* 82:70-77)). Protein concentrations were determined by reaction with bicinchoninic acid and copper sulfate (Deutscher (1990) *in Meth. Enzymol.*, vol. 182. San Diego: Academic Press, Inc.). During purification, CoADR activity in crude extracts was monitored by the NADPH and CoA disulfide dependent reduction of DTNB. DTNB and all substrates were added at 0.1 mM. For kinetic analysis of purified CoADR activity, the oxidation of NADPH was measured as the decrease in absorbance at 340 nm (De^{340} = 6,220 M^{-1} cm^{-1}), and was carried out in Tris-HCl (50 mM), pH 7.8, containing NaCl (50 mM).

ANALYSIS OF THIOLS FROM *S. AUREUS*. An analysis of the thiols produced by *S. aureus* was carried out by resuspending cell pellets (250 mg) in 50%

acetonitrile in 20 mM Tris-HCl, pH 8.0, containing monobromobimane (mBB) (2 mM) and incubating the suspension at 60°C for five minutes in the dark. Control samples are pretreated with N-ethylmaleimide (NEM) (2 mM) under the same conditions before the addition of mBB (to 2 mM). The cellular debris was removed by centrifugation, and the samples were diluted in 10 mM aqueous methane sulfonic acid for reverse phase HPLC analysis.

Example 1

Identification of a Coenzyme A Disulfide Reductase from *S. aureus*

In order to identify the enzyme responsible for maintaining CoA in its reduced form, *S. aureus* extracts were analyzed for a disulfide reductase specific for CoA disulfide. An overnight culture (10 ml) of R8325-4 was centrifuged (5,000 x g, 10 min), resuspended in 3 ml of TE buffer containing lysostaphin (5 mg/ml), and incubated at 37°C for 30 min until the suspension became viscous. Glass beads (1 g) and PMSF (to 1 mM) were added, the mixture was vortexed for two minutes and then centrifuged (14,000 x g, 10 min) to remove the insoluble cellular debris. The resulting viscous lysate was dialyzed exhaustively (3,600 M_r cutoff) against TE buffer. The dialysate was then assayed for the pyridine nucleotide (1 mM) and CoA disulfide (1 mM) dependent reduction of DTNB (1 mM). The results of this analysis are shown in Table 1.

Table 1. Identification of an NADPH and coenzyme A dependent oxidoreductase in extracts of *Staphylococcus aureus*. Shown is the ability of *S. aureus* extracts to reduce DTNB in the presence of various pyridine nucleotide and disulfide substrates.

	Disulfide				
Pyridine nucleotide	none	GSSG	cystine	pantethine	CoA disulfide
none	—	—	—	—	—
NADH	—	—	—	—	—
NADPH	+[a]	—	—	—	++++

[a] A very low NADPH dependent reduction of DTNB was detected in some extracts, which was attributed to the thioredoxin/thioredoxin reductase system present in most organisms.

Table 1 shows the ability of dialyzed *S. aureus* extracts to reduce DTNB in the presence and absence of NADH, NADPH and various disulfide substrates. DTNB was reduced only when the extracts are incubated with both CoA disulfide and NADPH. NADH, GSSG, cystine and pantethine did not function in the NADPH-dependent reduction of DTNB. DTNB is reduced only when the extracts are incubated with both CoA disulfide and NADPH. NADH cannot be used in the CoA disulfide dependent reduction of DTNB, nor can GSSG, cystine, or pantethine function in the NADPH dependent reduction of DTNB. These results indicate that *S. aureus* produces a Coenzyme A disulfide reductase (CoADR) that catalyzes specifically the reduction of CoA disulfide by NADPH. A low level of NADPH-dependent and CoA disulfide-independent reduction of DTNB was detected and was attributed to thioredoxin/thioredoxin reductase.

Example 2

Purification and Characterization of CoADR from *S. aureus*

A. PURIFICATION PROCEDURE. CoADR was fractionated from cellular extracts of *S. aureus* by following the NADPH- and CoA disulfide-dependent reduction of DTNB. An overnight culture of *S. aureus* strain R8325-4 grown in TSB (10 ml) at 37°C was used as an inoculum (0.4 ml) for each of ten 2L flasks containing TSB (1L). These cells were shaken (180 rpm) for 12h at 37°C before being harvested by centrifugation (7000 x g, 15 min). All subsequent handling of the sample prior to chromatography was carried out at 4°C. The cell pellet was resuspended in a minimum of TE buffer containing PMSF (1 mM) and lysostaphin (0.5 mg), incubated at 37°C with agitation for one hour (or until viscous), passed twice through a French pressure cell operating at 15,000 lb/in^2, and then centrifuged (15,000 x g, 20 min) to remove insoluble cellular debris. The supernatant was brought to 50% saturation with $(NH_4)_2SO_4$, stirred for 15 min, and centrifuged (15,000 x g, 10 min). The resulting supernatant was brought to 80% saturation with $(NH_4)_2SO_4$, centrifuged, and the pellet containing the CoADR activity was dissolved in a minimum of TE buffer containing PMSF (1 mM). The resulting solution was dialyzed exhaustively (3,500 M_r cutoff) against TE buffer containing PMSF (1 mM).

All chromatography was carried out at room temperature. The dialyzed $(NH_4)_2SO_4$ fraction was applied (1.0 ml/min) to an 2',5'-ADP-Sepharose® affinity column (1 by 5 cm) equilibrated with buffer A, which was Tris-HCl (20 mM), pH 8.0. The column was washed with buffer A (25 ml) and then eluted with a linear gradient (35 ml) of NaCl (0-4 M) in buffer A. The fractions (1 ml) exhibiting CoADR activity were pooled, concentrated and diluted in buffer A twice to reduce conductivity, and applied (1.0 ml/min) to a MonoQ HR 5/5 anion exchange column (1 ml) equilibrated with buffer A. The column was washed with 5 ml of buffer A and eluted with a linear gradient (25 ml) of NaCl (0.3-0.6 mM) in buffer A. The purity

of fractions showing CoADR activity was determined by SDS-PAGE (5% stacking gel; 12% separating gel) and silver staining.

A chart describing the purification of CoADR from 10 liters of *S. aureus* cells is shown in Table 2.

Table 2. Purification of Coenzyme A Disulfide Reductase from *Staphylococcus aureus*

Fraction	Total units[a]	Protein (mg)	Specific activity (units/mg)	Purification (X fold)	Yield (%)
soluble extract	3870	2560	1.51	1.0	100
50-80% amm. sulf. dialysate	2200	246	8.7	5.8	57
2'-5' ADP-sepharose	1800	0.84	2143	1420	47
MonoQ	960	0.21	4570	3030	25

[a] A unit was the amount of enzyme required to catalyze the reduction of 2 mmol DTNB (or 1 mmol of CoA disulfide) in 1 min.

As shown in Table 2, the primary purification step was the 2',5'-ADP-Sepharose affinity chromatography, which provided a 300-fold purification. 2',5'-ADP mimics NADPH, the enzyme's natural substrate for which it has micromolar affinity (*see*, Table 3). CoADR from the ADP column was contaminated by three other proteins that were easily removed by MonoQ anion exchange chromatography. CoADR activity eluted in two peaks. The second peak had a higher specific activity than the first and was the only fraction retained for further study. SDS-PAGE followed by silver staining of this fraction shows that it is greater than 95% homogeneous. All subsequent physical and chemical characterizations were performed on this sample.

B. DETERMINATION OF CoADR NATIVE MOLECULAR WEIGHT. The native molecular weight of CoADR was estimated by gel exclusion chromatography. A sample of the purified CoADR from the monoQ column (0.5 ml) was loaded onto a sepharose 6 HR 10/30 gel exclusion column (Pharmacia, 25 ml) (0.5 ml/min) equilibrated in Tris-HCl (20 mM), pH 8.0, containing NaCl (1 M) and then eluted isocratically in the same buffer. Fractions containing CoADR were identified by UV absorbance and activity measurements. The native molecular weight of CoADR was estimated by extrapolation of the parameter K from a standard plot of K versus the log of the molecular weight of protein standards. The parameter, K, is defined as $(V_e-V_o)/V_s$ where V_e is the volume of solvent required to elute the protein of interest, V_o is the void volume or the volume of solvent required to elute a totally excluded protein, and V_s is the volume of the stationary phase as determined by the subtraction of the void volume from the total volume of the column. Freifelder (1976) *Physical Biochemistry* (W.H. Freeman and Company. New York).

Purified CoADR migrates as a single polypeptide of ~50 kD apparent molecular weight according to SDS-PAGE. Native CoADR elutes between 44 and 158 kD from the superose gel filtration column. The K value calculated for CoADR (0.5) can be extrapolated to a molecular weight of approximately 85 kD. This suggests that CoADR is a homodimer in its native state.

C. FLAVIN COFACTOR IDENTIFICATION. The absorbance spectrum of purified CoADR is that of a typical flavoenzyme, having maxima at 454 and 360 nm. To identify the apparently bound flavin, purified CoADR was denatured, and the migration of the released cofactor on a reverse phase HPLC was compared to that of riboflavin, FMN, and FAD. A sample (0.1 ml) of CoADR (10 mM) was heated to 95°C for 10 min and then centrifuged (17,000 x g, 10 min) to remove the denatured protein. The supernatant was then separated by reverse phase HPLC. Sundquist et al. (1989) *J. Biol. Chem.* 264:719-725.

The visible absorbance spectrum of purified CoADR is typical of that of a flavoenzyme. The enzyme has a l_{max} at 450 nm and 360 nm. Boiled and centrifuged CoADR demonstrated no detectable CoADR activity and was not observable on an SDS-PAGE gel. The sample maintained the absorbance spectra of a flavin, suggesting that boiling had released the cofactor and centrifugation had removed the denatured protein. A chromatograph of the flavin sample separated by reverse phase HPLC showed that the flavin from CoADR migrates the same as FAD and elutes much later than either riboflavin or FMN. Thus, CoADR is a flavoenzyme utilizing a non-covalently bound FAD as cofactor. Quantitation of the flavin released from CoADR reveals that 1 flavin molecule is released per subunit of enzyme denatured.

D. THIOLS/ACTIVE SITE. To determine if CoADR utilized catalytic cysteine residues, a thiol titration of the active site was performed. A solution of oxidized CoADR (9.5 mM) in TE was incubated with NADPH (0.2 mM) for 10 min at ambient temperature before being diluted (1:1) with TE containing 8 M urea and DTNB (0.2 mM). The absorbance at 412 nm was then measured and compared to that of a similar reaction in which CoADR was not incubated with NADPH. The number of thiols liberated per FAD was then calculated.

Thiols are the most reactive nucleophiles in the cell. mBB is a very reactive electrophile and reacts with most cellular nucleophiles. NEM, however, is less reactive and more selective for thiols. NEM pretreatment of a sample thus selectively modifies thiols so that they do not react with mBB. Thiols are thus identified as peaks appearing in the mBB treated sample but not in the NEM pretreated sample. *S. aureus* produces predominantly CoA, H_2S, and a small amount of cysteine. 3'-Dephosphorylation of CoA occurs under the acidic conditions of the HPLC protocol, so CoA is determined by the combined peaks of CoA and 3'-dephospho-CoA. The majority of H_2S presumably originated from FeS proteins. A large peak running at 18 minutes has been previously isolated and characterized as bismethylbimane. This

compound apparently arises from the demethylation of mBB by some cellular factor. No GSH was detected.

Reduction of CoADR with NADPH liberated 3.2 ± 0.2 thiols/subunit (according to FAD concentration), while 0.9 ± 0.2 thiol was detected in the denatured enzyme that was not incubated with NADPH. This suggests that CoADR in its oxidized state has one reduced cysteine, likely buried in its core, and that upon reduction with NADPH a disulfide bond involving at least one enzymic cysteine is reduced. Thus, CoADR likely utilizes a thiol-disulfide exchange mechanism in its reduction of CoA disulfide. While this is suggestive of an active site having two cysteine residues, it only demonstrates that incubation with NADPH results in the reduction of a disulfide bond involving at least one enzymic thiol. Indeed, CoADR utilizes only a single active site cysteine, which in the oxidized enzyme forms a mixed disulfide with CoA.

E. Cu^{2+} CATALYZED OXIDATION OF CYSTEINE, GSH, AND CoA. To determine the relative stability of CoA to heavy metal-catalyzed oxidation, the rate of Cu^{2+} catalyzed oxidation of cysteine, GSH, and CoA were compared. Each sample (2 ml) of thiol (1 mM) in Tris-HCl buffer (20 mM), pH 7.5, containing $CuCl_2$ (1 mM) was incubated at ambient temperature. Thiol determination was then carried out at various times by adding aliquots (100 ml) from each sample to a solution (900 ml) of DTNB (1 mM) in Tris-HCl buffer (20 mM), pH 8.0, containing EDTA (1 mM). The absorbance of these samples at 412 nm was measured and the concentration of remaining thiol determined.

Although cysteine is necessary for all cells, it is rarely the predominant cellular thiol, especially in aerobic organisms. This is believed to be because cysteine undergoes rapid metal catalyzed autooxidation when exposed to oxygen to produce cystine and hydrogen peroxide.

$$2\text{Csy-SH} + Cu^{2+} + O_2 \rightarrow \text{Cys-SS-Cys} + Cu^{2+} + H_2O_2$$

Glutathione provides a reserve of cysteine which is much more stable to metal catalyzed oxidation. Although CoA would not be an efficient storage form of cysteine, as the predominant thiol in *S. aureus* it should be resistant to metal-catalyzed oxidation. Cys, GSH, and CoA each show a different rate of copper-catalyzed autooxidation. Cysteine is the least stable to oxidation. CoA and GSH are of comparable stability and are much more stable than cysteine. The high concentrations of CoA in *S. aureus* thus represent a stable thiol buffer analogous to that provided by GSH in other organisms.

F. KINETIC CHARACTERIZATION OF COADR SUBSTRATE SPECIFICITY. CoADR is specific for CoA disulfide and NADPH. The specificity of CoADR for these substrates and various other biological disulfides were quantitated kinetically. Kinetic measurements were performed in a 1 cm path-length quartz cuvette maintained at 37°C. Each assay (0.3 ml) was carried out in buffer A containing CoADR (2-10 nM), NADPH (2-200 μM), and either CoA disulfide (2-200 μM), 3'-dephospho-CoA disulfide (10-500 μM), 4,4'-diphosphopantethine (2-400 μM), pantethine (10 μM-100 mM), and glutathione disulfide (10 μM-100 mM), cystine (10 μM-100 mM), and CoA-glutathione mixed disulfide (10 μM-100 mM). Enzyme and NADPH were combined in buffer and equilibrated to 37°C, and the reaction was initialized by the addition of the disulfide substrate. The activity of CoADR was monitored at 340 nm as the decrease in absorbance resulting from the oxidation of NADPH. All kinetic measurements were recorded in the linear range, and at least seven substrate concentrations were used for each analysis. Kinetic constants were calculated from a linear least squares fit of the initial velocity data to the Michaelis-Menton equation using the program HyperO. Cleland (1979) *Meth. Enzymol.* 63:103-138. The results of this analysis are shown in Table 3.

A variation of the pH between 6.0 and 9.0 at constant NADPH and CoA disulfide concentrations, showed that CoADR has an optimal operating pH of 7.5-8.0. CoADR is very specific for its physiological substrates CoA disulfide and NADPH and is saturated by micromolar

concentrations of each. The K_m for NADPH, at saturating CoA disulfide, was 2 μM and the K_m for CoA disulfide, at saturating NADPH, was 11 μM. Table 3 shows the results of the kinetic analysis of the CoADR catalyzed reduction of various disulfide substrates by NADPH.

Table 3. Steady state kinetic analysis of the oxidation of NADPH by various disulfide substrates catalyzed by *S. aureus* coenzyme A disulfide reductase.[a]

Substrate	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m ($M^{-1}s^{-1}$) x 10^6
NADPH	1020 ± 60	1.6 ± 0.5	600 ± 100
CoA disulfide	1000 ± 200	11 ± 2	80 ± 10
3'-dephospho-CoA disulfide	1400 ± 200	140 ± 40	10 ± 2
4,4'-diphospho-pantethine	540 ± 40	80 ± 10	3.3 ± 0.4
pantethine	nd	nd	nd
CoASSG	800 ± 70	1100 ± 200	0.72 ± 0.08
cystine	nd	nd	nd
GSSG	nd	nd	nd

[a] CoA disulfide was maintained at 120 mM to determine the kinetic parameters for NADPH, and NADPH was maintained at 200 mM for the disulfide substrates.

nd = no activity detected.

A selective deletion of the chemical moieties that make up CoA has provided some insight to their contribution to the binding and turnover of CoA disulfide. Since hydride transfer from NADPH has been shown to be rate limiting for those pyridine nucleotide-dependent disulfide reductases

investigated to date, it is not surprising that the k_{cat} values for each of the substrates are similar. The 3'-phosphate moieties contribute predominantly to ground state binding (~ 1.5 kcal), resulting in a 10-fold increase in K_m with no detectable change in k_{cat} for 3'-dephospho-CoA disulfide as compared to CoA disulfide. It is interesting that the K_m for the 4,4'-diphosphopantethine is similar to the 3'-dephospho-CoA disulfide but that the k_{cat} is 2-3 fold lower. Thus, the adenyl moiety does not appear to contribute to ground state binding, but rather to transition state binding. In addition, the low k_{cat} for the 4,4'-diphosphopantethine suggests that disulfide reduction may be rate limiting for this substrate. Since pantethine is not turned over by CoADR, the 4- and 4'-phosphate moieties are clearly essential for substrate binding and turnover. Interestingly, CoADR did act on CoASSG although with a high K_m = 1.1 mM. No activity could be detected for GSSG or cystine.

Example 3

Recombinant Production of *S. aureus* CoADR

The gene encoding *S. aureus* CoADR was isolated and sequenced using methods described below. Generally, the gene was identified by PCR using degenerate primers shown in Table 4A based on the N-terminal sequence of CoADR and an internal amino acid sequence of the enzyme shown in Table 4B. The DNA fragment generated by the PCR was labeled and used as a probe in the isolation of a 4.5 kB *Hind*III fragment from *S. aureus* genomic DNA that carried the *cdr* gene. The sequence of the open reading frame and the deduced amino acid sequence are shown in FIG. 1 and FIG. 2, respectively.

Table 4A. Degenerate oligonucleotide primers used in the PCR amplification of an internal region of the gene encoding CoADR

Oligomer	Sequence
SD-111 (N-terminal region)	5'-GG(AT)GC(AT)GT(ACT)GC(AT)GG(AT)GG(AT)GC-3' (SEQ ID NO:___)
SD-113 (internal region)	5'-AAG(AT)G(CA)AAATAG(AG)TTAATAG(AG)TT(AT)AT(AT)CCAAC-3' (SEQ ID NO:___)

Table 4B. Degenerate oligonucleotide primers used in the PCR amplification of an internal region of the gene encoding CoADR

Region	Sequence
N-terminal Peptide	PPKIVVVGAVAGGAT (SEQ ID NO:___)
Internal Peptide	NQPILDESDKREIPYP (SEQ ID NO:___)

Identification of a DNA Fragment Encoding the N-terminal of CoADR — CoADR was purified as described in Example 2. CoADR and CoADR cleaved with cyanogen bromide, Matsudaira (1990) *Meth. Enzymol.* 182:602-613, were separated by SDS-PAGE, blotted onto Immobilon PVDF membrane (Millipore), and visualized with coomasie blue staining (in the absence of acetic acid). The bands corresponding to native CoADR and a 35 kD CNBr cleavage product were excised and submitted for N-terminal sequencing to the Protein Sequencing Laboratory of the University of Victoria (Victoria, British Columbia). The N-terminal amino acid sequences of the native CoADR and of the 35 kD CNBr-cleavage product are shown in Table 4B.

Degenerate oligonucleotides, which were designed to encode the N-terminal (coding) and internal (non-coding) sequences, were used as

primers for the PCR of *S. aureus* genomic DNA. The PCR reaction contained 10 ng genomic DNA, 100 pmol each of 5'-GG(AT)GC(AT)GT(ACT)GC(AT)GG(AT)GG(AT)GC-3' (SEQ ID NO:___) and 5'-AAG(AT)G(CA)AAATAG(AG)TTAATAG(AG)TT(AT)AT(AT)CCAAC-3' (SEQ ID NO:___), $MgCl_2$ (2.4 mM), tetramethyl ammonium chloride (Sigma) (60 mM), deoxynucleotide triphosphates (dNTPs) (0.25 mM of each), and 1X PCR buffer (Gibco BRL). The reaction was incubated at 95°C (30 sec), 47°C (30 sec), and 72°C (30 sec) for 30 cycles. The resulting 600 bp PCR product was cloned directly using a TA cloning kit (Invitrogen) and sequenced using the universal "forward" and "reverse" primers which are homologous to the flanking region of the multiple cloning site within the plasmid pCR II (the TA cloning vector (Invitrogen)).

Cloning and Sequencing of the Gene Encoding CoADR — The cloned PCR fragment encoding the N-terminal of CoADR was excised from the TA cloning vector by digestion with *Eco*RI and band purified from an agarose gel. The fragment was labeled with digoxygenin and used to probe Southern blots of *S. aureus* genomic DNA digested with various restriction enzymes. A single 4.5 kB *Hind*III fragment that hybridized to the probe under stringent conditions (68°C, 0.1 SSC buffer containing 0.1% SDS) was subcloned into plasmid pUC18 and sequenced. Initial sequencing primers were designed to prime within the sequence of the PCR fragment described above and to sequence into the flanking region. New primers were designed within the new sequences and the nucleotide sequence of the entire gene was thus determined stepwise. All of the sequences were confirmed by sequencing both the coding and noncoding strands.

Heterologous Overexpression of CoADR in E. coli — The open reading frame encoding CoADR was amplified by the PCR using the N-terminal PCR primer GGGAATTCCATATGCCCAAAATAGTCGTAGTCGG, (SEQ ID NO:___), and the C-terminal PCR primer

CCCAAGCTTTATTTAGCTTTGTAACCAATCAT (SEQ ID NO:___). The resulting fragment was digested with *Nde*I and *Hind*III, purified, and ligated with pET22B(+) (Novagen, Madison WI) that had been digested with the same two enzymes and purified similarly to produce plasmid pCDRX. An overnight culture (10 mL) of *E. coli* BL21 (DE3) cells harboring pCDRX was washed twice in 10 mL of TB medium, and used as an inoculum for 1 liter of the same medium containing ampicillin (400 mg/mL). The resulting culture was incubated at 37°C until it reached mid-stationary phase ($A_{600\ nm}$ = 1.2), induced to express recombinant CoADR (rCoADR) by the addition of IPTG (to 1 mM), and then incubated for an additional 3 hours at 37°C. The cells were harvested and the recombinant enzyme was purified as described in Example 1 for native CoADR except that lysozyme (2 mg/mL) was used in place of lysostaphin to assist in disrupting the cells. The purity of the resulting recombinant enzyme was measured by SDS-PAGE and staining with brilliant blue. The specific activity and purity from *E. coli* glutathione reductase was measured spectrophotometrically by following the oxidation of NADPH colorimetrically. This procedure allows for the recovery of approximately 10 mg/mL of rCoADR from the soluble fraction of the cell lysate that is >98% pure and free of glutathione reductase activity.

Gene Inactivation - A *cdr*⁻ strain of *S. aureus*, strain RN4220, was created by the recombination of a plasmid carrying an internal fragment of the *cdr* gene into the RN4220 chromosome by Cambell-like integration. The resultant mutant formed small colonies on TSA plates and had less than 10% recovery from starvation conditions.

Thus, an isolated *S. aureus* CoADR polypeptide, and DNA that encodes an *S. aureus* CoADR are provided herein. Although preferred embodiments of the subject invention have been described in some detail, it is understood that obvious variations can be made without departing from the spirit and the scope of the invention as defined by the appended claims.

WE CLAIM:

1. An isolated *Staphylococcus aureus* Coenzyme A disulfide reductase (CoADR) polypeptide.

2. The CoADR of claim 1 comprising the amino acid sequence depicted in Figure 2 (SEQ ID NO:___).

3. An isolated polynucleotide encoding the *S. aureus* CoADR of claim 1.

4. An isolated polynucleotide encoding the *S. aureus* CoADR of claim 2.

5. An expression vector comprising the polynucleotide of claim 3 operably linked to control sequences that direct the transcription of the polynucleotide whereby said polynucleotide is expressed in a host cell.

6. A host cell comprising the expression vector of claim 5.

7. A method for producing a *S. aureus* Coenzyme A disulfide reductase comprising:

culturing the host cell of claim 6 under conditions that allow the production of the Coenzyme A disulfide reductase; and

recovering the Coenzyme A disulfide reductase.

8. A probe useful for detecting the presence of a polynucleotide encoding a *Staphylococcus aureus* CoADR, comprising an oligonucleotide of at least eight nucleotides capable of specifically hybridizing to the polynucleotide under appropriate stringency condition.

9. An antibody reactive with a *Staphylococcus aureus* Coenzyme A disulfide reductase (CoADR).

10. The antibody of claim 9, wherein said antibody is a monoclonal antibody.

11. The antibody of claim 9, wherein said antibody is a polyclonal antibody.

12. A method for identifying a compound that modulate *S. aureus* Coenzyme A disulfide reductase (CoADR) activity, comprising:

(a) providing an *S. aureus* CoADR capable of catalyzing the specific reduction of Coenzyme A disulfide to Coenzyme A with the concomitant oxidation of NADPH to $NADP^+$;

(b) contacting a test compound with the CoADR; and

(c) measuring the effect of the test compound on the CoADR activity, thereby identifying a compound that modulates *S. aureus* CoADR activity.

13. A method of treating a Gram-positive bacterial infection in an infected individual, comprising administering to such individual an effective antimicrobial amount of a compound identified by method of claim 12.

14. The method of claim 13, wherein the infection is a *Staphylococcus aureus* infection.

15. The method of claim 14, wherein the compound is a pantethine derivative.

16. A method of detecting *S. aureus* in a sample containing or suspected to contain *S. aureus*, comprising:

(a) contacting the sample with the oligonucleotide probe of claim 8, thereby forming form a hybrid complex;

(b) detecting the presence of a hybrid complex; and

(c) correlating the presence of the hybrid complex with the presence of *S. aureus* in the test sample.

17. A method of detecting *S. aureus* in a sample containing or suspected to contain *S. aureus*, comprising:

(a) contacting the sample with the antibody of claim 9, thereby forming an antibody-CoADR complex;

(b) detecting the presence of the complex; and

(c) correlating the presence of the complex with the presence of *S. aureus* in the test sample.

18. A method of detecting *Staphylococcus aureus* in a sample containing or suspected to contain *S. aureus*, comprising:

(a) incubating the test sample with a composition comprising a substrate which, when catalytically activated by *S. aureus* Coenzyme A disulfide reductase (CoADR), produces a detectable signal;

(b) detecting the presence of the signal; and

(c) correlating the presence of the signal with the presence of *S. aureus* in the test sample.

19. A method for isolating a Coenzyme A disulfide reductase (CoADR) polypeptide from a bacterial cell medium containing the CoADR polypeptide, wherein the method comprises:

(a) performing a protein precipitation step with the bacterial cell medium to yield a first CoADR mixture;

(b) subjecting the first CoADR mixture to an affinity purification step to yield a second CoADR mixture;

(c) performing anion exchange chromatography on the second CoADR mixture to yield a third CoADR mixture, wherein the third CoADR mixture has a greater concentration of CoADR than the bacterial cell medium CoADR mixture.

20. The method of claim 19, wherein the precipitation step comprises adding to the bacterial cell medium ammonium sulfate to 50% saturation to yield a first precipitate and a first supernatant, recovering the first supernatant, adding ammonium sulfate to 80% saturation to yield a second precipitate and a second supernatant, and recovering the second precipitate.

21. The method of claim 19, wherein the affinity purification step is performed using a 2',5'-adenosine diphosphate affinity matrix.

22. The method of claim 19, wherein the anion exchange step is performed using a strong anion exchange matrix.

23. The method of claim 20, wherein the affinity purification step is performed using a 2',5'-adenosine diphosphate affinity matrix and the anion exchange step is performed using a strong anion exchange matrix.

24. A *Staphylococcus aureus* Coenzyme A disulfide reductase produced by the method of claim 19.

25. A diagnostic test kit comprising:

(a) an oligomer capable of specifically hybridizing to a polynucleotide encoding CoADR, or a portion thereof; and

(b) instructions for conducting the diagnostic test.

26. A diagnostic test kit comprising:

(a) an antibody capable of specifically binding to an *S. aureus* CoADR polypeptide; and

(b) instructions for conducting the diagnostic test.

27. A diagnostic test kit comprising:

(a) an *S. aureus* CoADR polypeptide; and

(b) instructions for conducting the diagnostic test.

28. A compound that inhibits or kills a microorganism, wherein said compound effectively inhibits Coenzyme A disulfide reductase activity of said microorganism.

29. The compound of claim 28, wherein said microorganism is a Gram-positive microorganism.

30. The compound of claim 29, wherein said Gram-positive microorganism is *Staphylococcus* spp.

31. The compound of claim 29, wherein said Gram-positive microorganism is *Enterococcus* spp.

32. A method of treating an individual suspected of having an infection due to a microorganism, comprising administering to said individual, the compound of claim 25 in a pharmaceutically acceptable excipient, in an amount effective for inhibiting or killing said microorganism.

ATGCCCaAAATAGTCGTAGTCGGAGCAGTCGCTGGTGGTGCAACATGTGCCAGCCAAATTCGACGTTTAGATAAAGAAAGTGACATT
ATTATTTttGAAAAAGATCGTGATATGAGCTTTGCTAATTGTGCATTGCCTTATGTCATTGGCGAAGTTGtTGAAGATAGAAGATAT
GCTTTAGCGTATaCACCtGAAAAATTTTATGATAGAAAGCAAATTACAGTAAAAACTTATCATGAaGTTATTGCAATCAATGATGAA
AGACAAaCTGTATCTGTATTAAATAGAAAGACAAACGAACAAtTTGAAGAATCTTACGATAAACTCATTTTAAGCCCTGGTGCAAGT
GCAAATAGCCTTGGCTTtGAaAGTGATATTACATTCACACTTAGAAATTTAGAAGACACTGATGCTATCGATCAATTCATCAAAGCA
AATCAaGTTGATAAAGTATTGGTTGTAGGTGCAGGTTATGTTTCATTAGAAGTtCTTGAAAATCTTaATGAACGTGGTttTACACCCT
ACTtTAATTCATCGATCTGATAAGATAAATTAAATGGATGCCGACATGAATCAACCTATACTTGATGAATTAGATAAGCGGGAG
ATTCCATACCGTTTAAATGAGGAAATTAATGCTATCAATGGAAATGAAATTACATTTAAATCAGGAAAAGTTGAACATTACGATATG
ATTATTGAAGGTGTCGGTACTCACCCCAATTCAAAATTTATCGAAAGTTCAAATATCAAACTTGATCGAAAAGGTTTCATACCGGTA
AACGATAAATTTGAAACAAATGTTCCAAACATTTATGCAATAGGCGATATTGCAACATCACATTATCGACATGTCGATCTACCGGCT
AGTGTTCCTTTAGCTTGGGGCGCTCACCGTGCAGCAAGTATTGTTGCCGAACAAATTGCTGGAAATGACACTATTGAATTCAAAGGC
TTCTTAGGCAACAATATTGTGAAGTTCTTTGATTATACATTTGCGAGTGTCGGCGTTAAACCAAACGAACTAAAGCAATTTGACTAT
AAAATGGTAGAAGTCACTCAAGGTGCACACGCGAATTATTACCCAGGAAATTCCCCTTTACACTTAAGAGTATATTATGACACTTCA
AACCGTCAGATTTTAAGAGCAGCTGCAGTAGGAAAAGAAGGTGCAGATAAACGTATTGATGTACTATCGATGGCAATGATGAACCAG
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AAAGCTAAAtAA

FIG. 1

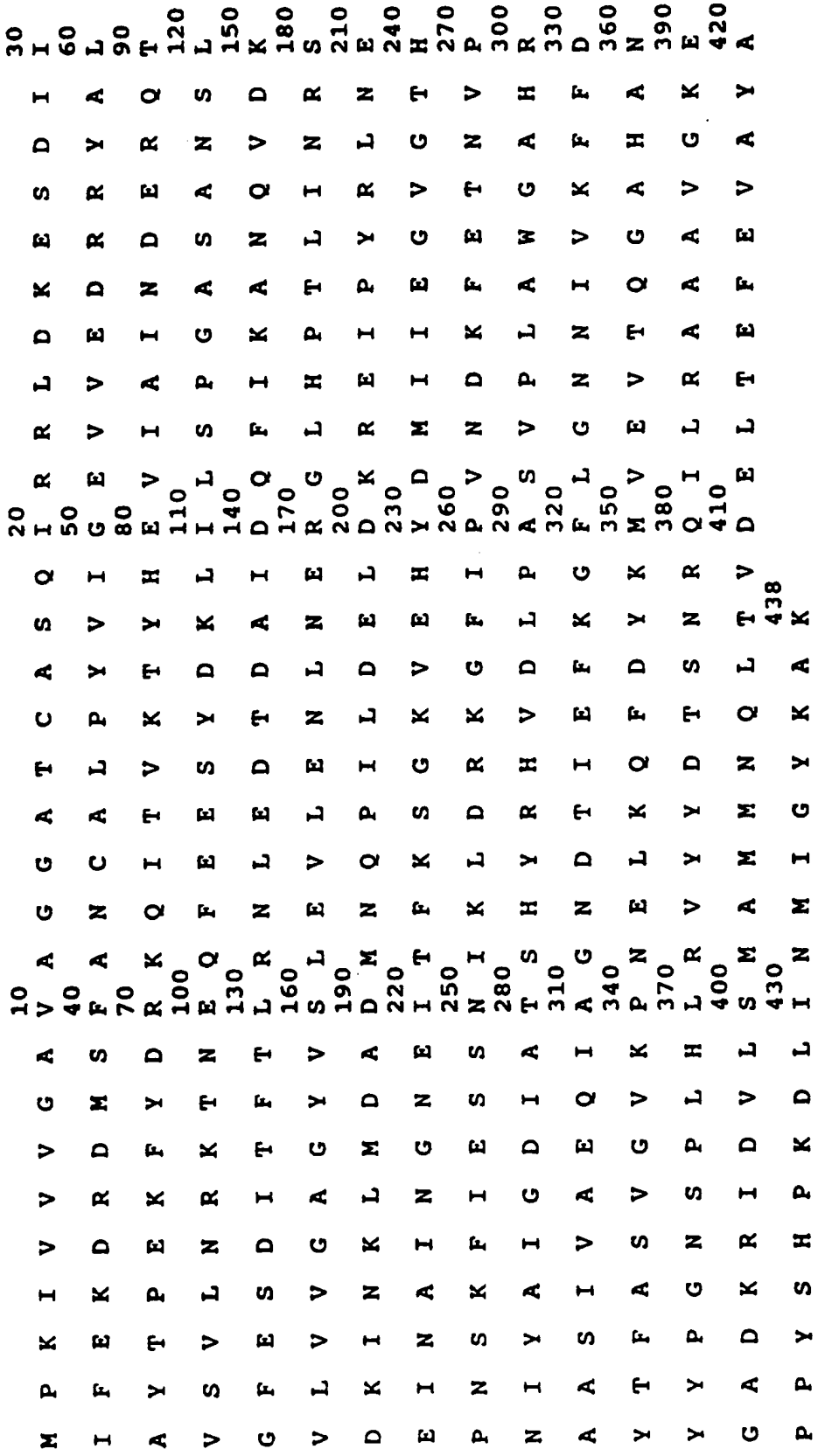
10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 200 210 220 230 240 250 260 270 280 290 300 310 320 330 340 350 360 370 380 390 400 410 420 430 438
M P K I V V V G A V A G G G A T C A S Q I R R L D K E S D I I
I F E K D R D M S F A N C A L P Y Y V I G E V V E D R R Y A L
A Y T P E K F Y D R K Q I T V K T Y H E V I A I N D E R Q T
V S V L N R K T N E Q F E E S Y D K L I L S P G A S A N S L
G F E S D I T F T L R N L E D T D A I D Q F I K A N Q V D K
V L V V G A G Y V S L E V L E N L N E R G L H P T L I N R S
D K I N K L M D A D M N Q P I L D E L D K R E I P Y R L N E
E I N A I N G N E I T F K S G K V E H Y D M I I E G V G T H
P N S K F I E S S N I K L D R K G F I P V N D K F E T N V P
N I Y A I G D I A T S H Y R H V D L P A S V P L A W G A H R
A A S I V A E Q I A G N D T I E F K G F L G N N I V K F F D
Y T F A S V G V K P N E L K Q F D Y K M V E V T Q G A H A N
Y Y P G N S P L H L R V Y Y D T S N R Q I L R A A A V G K E
G A D K R I D V L S M A M M N Q L T V D E L T E F E V A Y A
P P Y S H P K D L I N M I G Y K A K

FIG. 2

INTERNATIONAL SEARCH REPORT

Inter onal Application No
PC1/US 96/20017

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/53 C12N9/02 C12N1/21 C07K16/40 C12Q1/68
G01N33/50 G01N33/53 G01N33/569 A61K31/16

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K C12Q G01N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	96TH GENERAL MEETING OF THE AMERICAN SOCIETY FOR MICROBIOLOGY, NEW ORLEANS, LOUISIANA, USA, MAY 19-23, 1996. ABSTRACTS OF THE GENERAL MEETING OF THE AMERICAN SOCIETY FOR MICROBIOLOGY 96 (0). 1996. 263. ISSN: 1060-2011, XP000671809 DELCARDAYRE S B ET AL: "Purification and characterization of a coenzyme A disulfide reductase from Staphylococcus aureus." abstract no. D-118 see abstract ---	1-8

☐ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
7 May 1997	20.05.97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Hornig, H

INTERNATIONAL SEARCH REPORT

ernational application No.
PCT/US 96/ 20017

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 13-15,32
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 13-15,32 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.